Contents

Supplemental methods

A. Intra- and inter- assay performance

Daily intra-assay performance was evaluated by calculating the coefficient of variation in duplicate or triplicate wells. Samples with > 15% variation were repeated by ELISA. Inter-assay reproducibility was assessed by creating a "positive control" and "negative control" master-mix comprised of patients who were noted to have high or low titers in preliminary studies. The "positive" and "negative" controls were made by mixing equal volumes from patients with high (n=3, 40 µl of each sample) and low (n=4, 30 µl of each sample) reactivity in the PRT/H ELISA, respectively. The same positive and negative master-mix (diluted 1:500) was utilized on all PRT/H ELISA plates described in this report. The results of the positive and negative controls were monitored and assays were repeated if the positive/negative controls deviated from the mean positive or negative of aggregate runs by ± 0.5. ELISA reproducibility was also verified by repeating plates on different dates. Results of the positive/negative controls on each ELISA plate and the correlation of results from repeat ELISAs are shown in Figure S1.

B. ^{14}C Serotonin release assay methods

For the serotonin release assay, washed platelets labeled with ^{14}C-serotonin were incubated with or without protamine (1.25 µg/mL). Heat-inactivated plasma samples (diluted 1:40) from individual CPB patients demonstrating high positivity in the screening ELISA (A_{450nm} > 3) were incubated with the ^{14}C-serotonin labeled platelets. Platelets were

incubated for 45 minutes at room temperature with heparin at final concentrations of 0 U/mL, 0.25 U/mL, 1 U/mL, 100 U/mL, or with heparin 1 U/mL and IV.3 (an anti-human CD32 to measure FcγRII dependent binding). After centrifugation, ^{14}C-serotonin release was measured by scintillation counting. Platelet activation was reported as % release of labeled ^{14}C-serotonin.

C. Absorbing sera on PRT and testing unbound antibody on PRT/H

Sera from patients expressing high-titer antibodies (mean A_{450nm} > 3.0, n = 32) were incubated on microtiter plates coated with protamine (31 ug/mL) at dilutions yielding 50% maximal binding (as determined by titration assays). After 1 hour incubation, unbound antibody was removed and placed on microtiter plates coated with both protamine (31 ug/mL) and PRT/H (31 ug/mL: 4 U/mL) for an additional 1 hour. An anti-human IgG gamma peroxidase (1:2500 dilution; Sigma, St Louis, MO) was used for detection of human antibodies with subsequent color development using TMB peroxide substrate (KPL, Gaithersburg, MD). Absorbance was measured at 450_{nm} using Spectramax 384 PLUS (Molecular Devices, Sunnyvale, CA) and results were analyzed using SoftMax PRO software (Molecular Devices). Results are shown in Figure S3.

D. Statistical analysis

In vitro antibody binding was compared using Student's t-test. Correlation of PRT/H and PF4/H antibody levels was tested with Pearson's correlation coefficient. Patient characteristics were compared by chi-squared or Student's t-test as appropriate.

Spearman's rank correlation was used to relate platelet counts to PRT/H antibody levels at the three sampling timepoints. To test clinical outcomes in association with seropositivity, antibody levels (absorbance) were analyzed both as a parametrically distributed continuous variable following logarithmic transformation and dichotomously as a positive or negative result with a cut-off absorbance of 1.2. Logistic regression modeling was used to test for an association between logarithmically transformed absorbance or seropositivity and perioperative morbidity (prolonged hospital stay or major adverse cardiac event before 30 days) with results expressed as an odds ratio with 95% confidence limits. A Cox proportional hazards model was used to test for association between antibody level and event (long-term major adverse cardiac event) free survival and was presented as a hazard ratio with 95% confidence limits. A multivariable logistic regression model was used to examine the association between patient characteristics and seropositivity at day 30. All patient characteristics in Supplemental Table 2 were tested; those variables with p values < 0.1 in the multivariable analysis were retained in the final model. A secondary analysis identified patients seropositive for both PRT/H antibodies ($A_{450nm} \geq 1.2$) and PF4/H antibodies ($A_{450nm} \geq 0.4$). Those seropositive for both ("double positive") were tested as above for associations with perioperative morbidity and event (major adverse cardiac event) free survival. Statistical analyses of in vitro data were performed using GraphPad Prism (Graph Pad Software Version 4.03); clinical data were analyzed with SAS version 9.2 (Cary, NC.). Differences were considered significant at $p < 0.05$. For this exploratory analysis, Bonferroni correction was not applied.

Supplemental tables

A. Table S1: Patient demographics

Patient Characteristics	**n = 500**
Age – year (mean ± SD)	63.2 ± 11.3
Female – no. (%)	142 (29)
Diabetes – no. (%)	154 (31)
Insulin Dependence	70 (14)
Coronary artery disease risk factors – no. (%)	
Smoking	100 (20)
Hypertension	483 (82)
Previous myocardial infarction	219 (37)
Congestive heart failure	214 (43)
Peripheral vascular disease	54 (11)
Hannan Score	0.036
Pumptime – minutes (mean ± SD)	146 ± 56

SD, standard deviation

B. Table S2: Clinical outcomes related to PRT/H antibody status at baseline

	Baseline protamine/heparin antibody status		
	$A_{450nm} \leq 1.2$	$A_{450nm} > 1.2$	p-value
Age – year (SD)	63 (12)	61 (11)	0.8
Female (%)	28.2	54.6	0.06
Weight –kg (SD)	86.6 (19.8)	88.7 (32.1)	0.75
Smoking (%)	20	0	0.11
Serum creatinine, preoperative	1.19 (0.88)	1.60 (1.93)	0.18
Previous myocardial infarction (%)	36.6	70	0.01
Chronic obstructive pulmonary disease (%)	23.3	27.3	0.76
Renal disease (%)	7	20	0.09
Hypertension (%)	82	90	0.51
Congestive heart failure (%)	44	30	0.38
Platelet count (SD)	295 (110)	284 (47)	0.49
Thrombosis (%)	4.5	0	0.49
Peripheral vascular disease (%)	10	40	0.003
Diabetes (%)	27.7	54.6	0.05
Insulin dependent diabetes (%)	11.6	55.6	< 0.0001
Hannan Score (SD)	0.04 (0.03)	0.04 (0.02)	0.98

SD, standard deviation

C. Table S3: Adverse clinical outcomes related to PRT/H antibody status

		PRT/H (+) v PRT/H (-), Day 0	PRT/H (+) v PRT/H (-), Day 3-7	PRT/H (+) v PRT/H (-), Day 30
Length of stay >10 days or in hospital death	p value	0.63	0.43	0.75
	Hazard ratio (95% CI)	1.07 (0.82-1.38)	0.92 (0.73-1.14)	0.97 (0.80-1.17)
Event-free survival (days to death, repeat cardiac surgery, myocardial infarction or need for myocardial revascularization)	p value	0.07	0.28	0.13
	Hazard ratio (95% CI)	1.65 (0.96-2.82)	1.26 (0.82-1.92)	1.17 (0.96-1.44)

PRT/H, protamine/heparin antibody; CI, confidence interval

D. Table S4: Multivariable logistic regression model predicting seropositivity at day 30

Predictor	P value	Odds Ratio	95% Confidence Limits on OR	
Age	0.003	0.97	0.96	0.99
Smoking	0.019	1.90	1.11	3.23
CHF	0.064	0.67	0.44	1.02
Diabetes	0.012	1.72	1.13	2.63
Hannan score	0.056	1.06	0.10	1.13

OR, odds ratio; CHF, congestive heart failure

Supplemental figures

A. Figure S1: Intra- and inter-assay validation

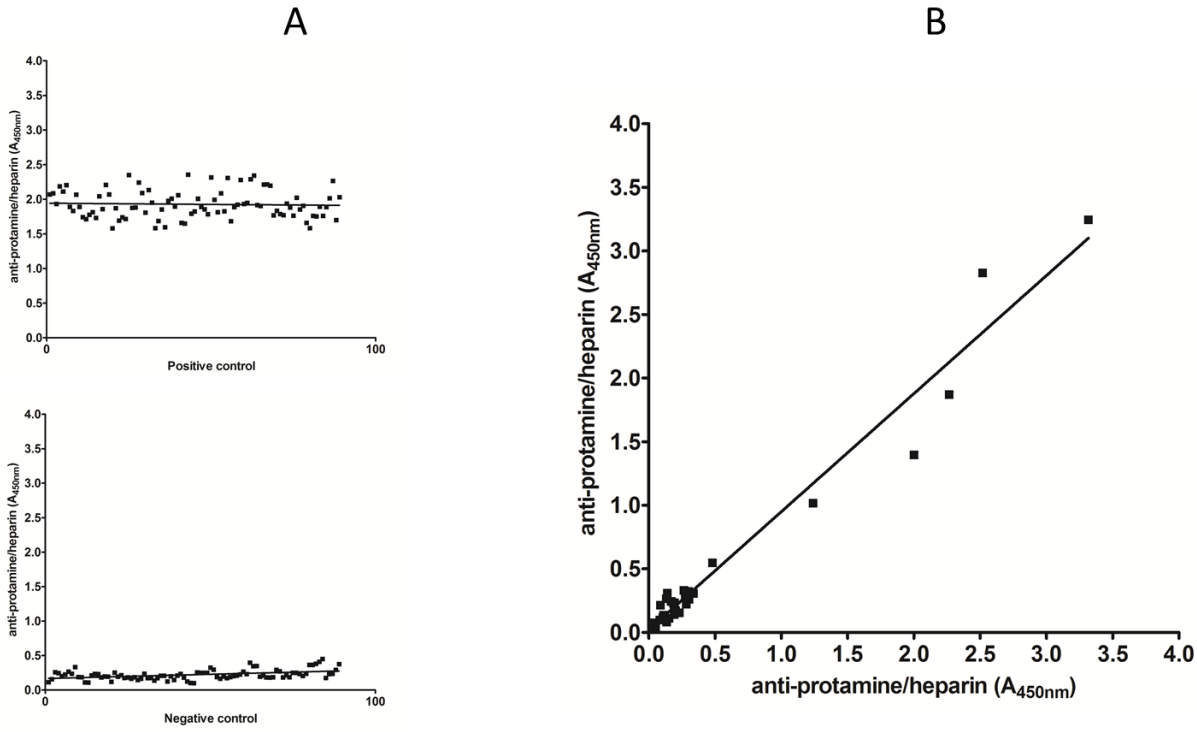

Figure S1: Intra- and inter-assay validation (A) Results of positive and negative control on each ELISA plate. (B) Results from repeating random samples at two separate timepoints ($R^2 = 0.9652$).

B. Figure S2: Platelet counts of PRT/H seronegative and seropositive individuals by timepoint

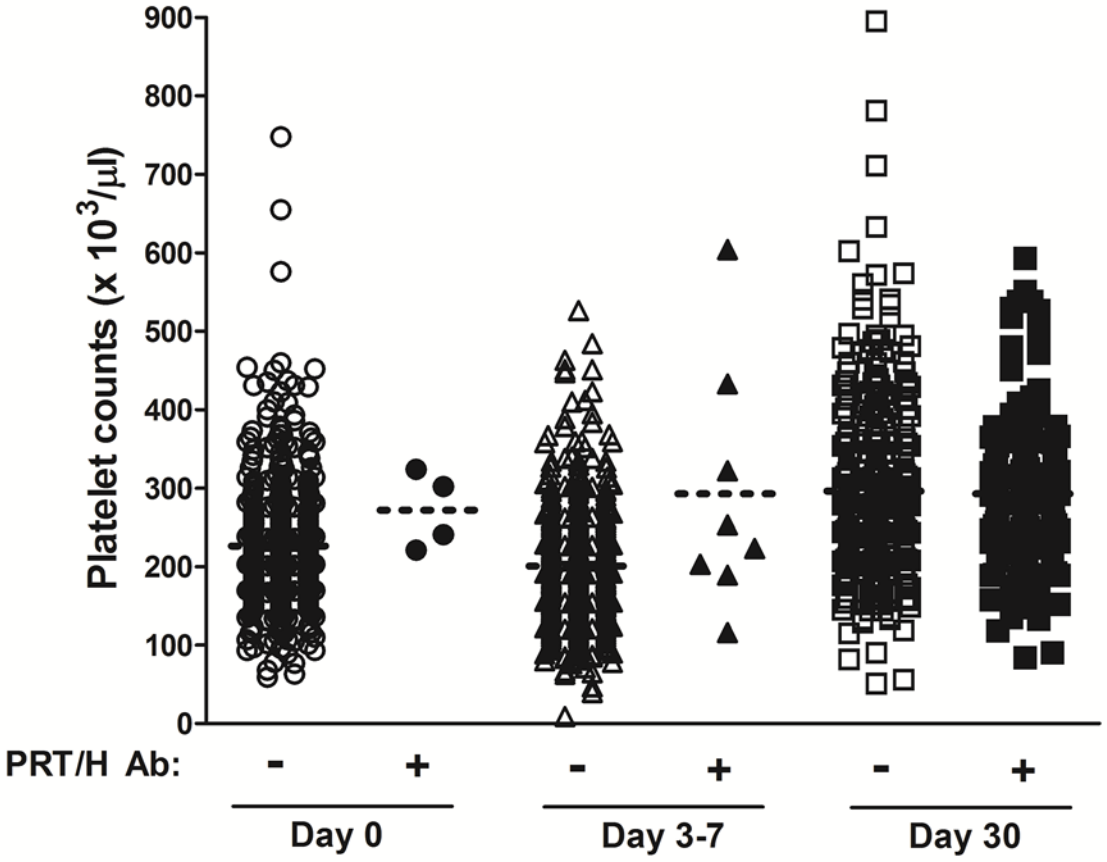

Figure S2: Platelet counts of PRT/H seronegative and seropositive individuals by time point. Platelet counts were obtained at time of antibody determination at baseline (day 0), day 3-7 and day 30. Cohorts are shown as PRT/H antibody (PRT/H Ab) negative ("-") or positive ("+"). Mean values of platelet counts are shown as dashed lines.

C. Figure S3: Antibody elutes on PRT and PRT/H

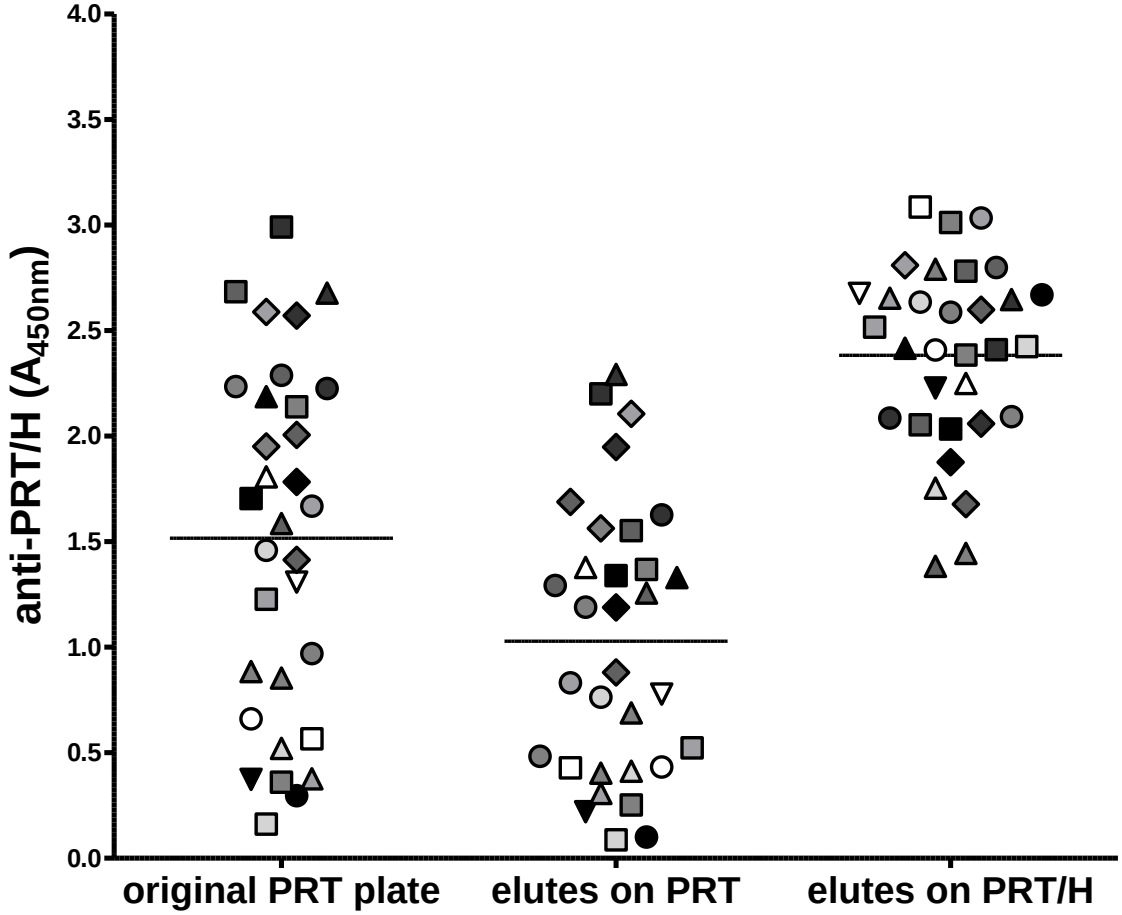

Figure S3: Sera absorbed on PRT and antibodies eluted onto PRT vs PRT/H. Sera from patients with high titer PRT/H antibodies (mean A_{450nm} > 3.0, n = 32) was first absorbed onto microtiter plates coated with PRT. Non-bound antibodies were then eluted off onto plates coated with both PRT and PRT/H.